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14. ABSTRACT

Mesenchymal stem cells (MSCs) have been proposed to be cellular vehicles for the targeted delivery and local production of biological agents in tumors. In this proposal we will stably transfect mesenchymal stem cells with a lentiviral vector containing a therapeutic gene and dual reporter gene mrfp-ttk. Specific Aims: 1) We will monitor breast cancer tropism of mesenchymal stem cells by multimodality imaging techniques; 2) We will demonstrate the ability of mesenchymal stem cells to target delivery of gene therapeutics to breast cancer in vitro; 3) We will determine the effect of mesenchymal stem cell to target delivery of gene therapeutics to breast cancer lung metastasis. Major findings from year 1 studies: 1) MSCs home to both subcutaneous breast cancer and its lung metastasis; 2) MSCs home to both premature and well-established breast cancer lung metastasis; 3) MSCs proliferate at tumor site; 4) MSCs show dissimilar differentiation potential at lung tumor and subcutaneous tumor niches; 5) lung tumor microenvironment upregulates BMP-2 and Noggin transcription, which favor MSCs osteoblastogenic differentiation.

15. SUBJECT TERMS
None listed.

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INTRODUCTION

We aimed to investigate the tropism capacity of mesenchymal stem cells (MSCs) to tumor environment. We thus labeled MSCs with the firefly luciferase (fLuc) and the green fluorescence protein (GFP) genes to monitor MSCs in vivo by bioluminescence imaging (BLI) and in tissue sections by emitted fluorescence, respectively. We found that MSCs homing to breast cancer lung metastasis differentiate into osteoblasts, whereas MSCs homing to subcutaneous breast cancer differentiate into adipocytes. The data provide evidence that MSCs home and engraft to tumor site, and that different tissue-tumor niche have distinct effect on MSCs differentiation. These finding may be clinically relevant because the beneficial effects of MSCs are being tested clinically in attempts to improve hematopoietic engraftment [1], to treat osteogenesis imperfect [2], graft-versus-host disease [3], and autoimmune diseases [4, 5], and to deliver therapy for malignancies [6, 7].

BODY

4T1 murine mammary adenocarcinoma cells were transduced with a lentivirus (pFU vector) carrying a bi-fusion reporter consisting of a humanized Renilla luciferase and a monomeric red fluorescent protein (ubiquitin-hRluc-mRFP) and FACS sorted to establish the 4T1-hRluc-mRFP cells (**Fig. 1**).

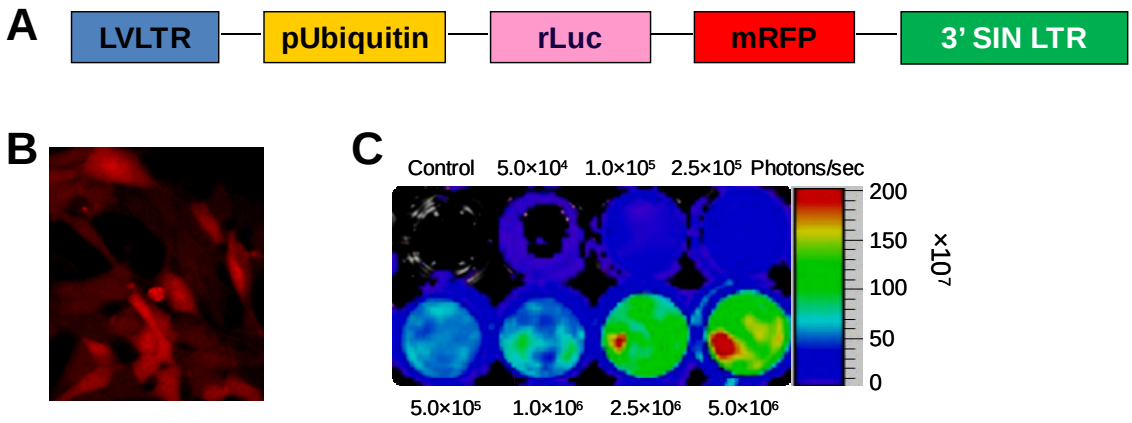


Fig. 1. Transfection and characterization of dual-reporter gene labeled murine breast cancer 4T1 cells (4T1-rLuc-mRFP). (***A***) Gene structure of lentivirus carrying dual-reporter (rLuc-mRFP) gene driven by a constitutive human ubiquitin promoter. (***B***) Fluorescence microscope image demonstrated that 4T1-rLuc-RFP cells uniformly express RFP in cytosol. (***C***) 4T1-rLuc-RFP cells constitutively express renilla luciferase.

Bone marrow-derived mesenchymal stem cells (MSCs) were harvested from femurs and tibias of L2G85 transgenic mice, constitutively expressing eGFP and firefly luciferase (fLuc). The MSC cell surface marker expression profile was tested by FACS and found to be CD90(+)CD40(+)CD11(+)CD106(-)CD34(-)CD45(-)CD117(-). The mesenchymal lineage of the murine MSCs was confirmed by their ability to readily differentiate into adipocytes and osteoblasts [8] (**Fig. 2**).

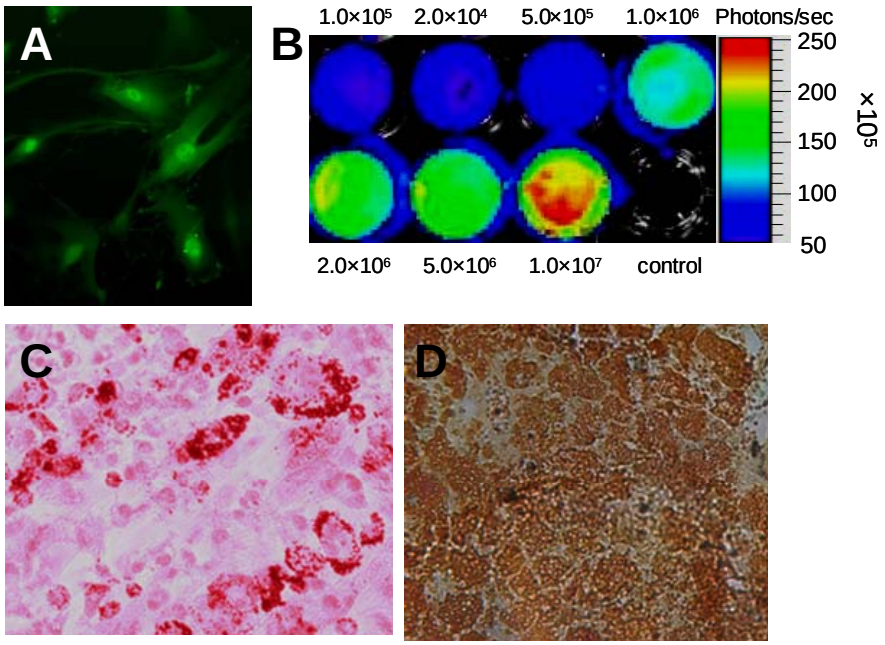


Fig. 2. Characterization of MSC-L2G85. (***A***) MSC-L2G85 cells constitutively express firefly luciferase. (***B***) Representative BLI images of MSC-LG85 cells. ***(C-D)*** Differentiation of MSC-LG85 cells into adipocytes as assessed by Oil O red staining ***(C)*** and Alizarin S staining (***D***), respectively.

To study the breast cancer tropism of MSCs, we designed the following experiment with 5 groups of mice: group 1, tail vein injection of 2 x10^5 4T1 murine breast cancer cells to Balb/C mice and followed the tumor growth without administration of MSCs; group 2, Balb/C mice administered with MSCs only; group 3, 4T1 cells and MSCs were intravenously co-injected; group 4, MSCs were administered 4 days after tail vein injection of 4T1 cells; group 5, MSCs were administered 7 days after subcutaneous inoculation of 10^6 4T1 cells (**Fig. 3**).

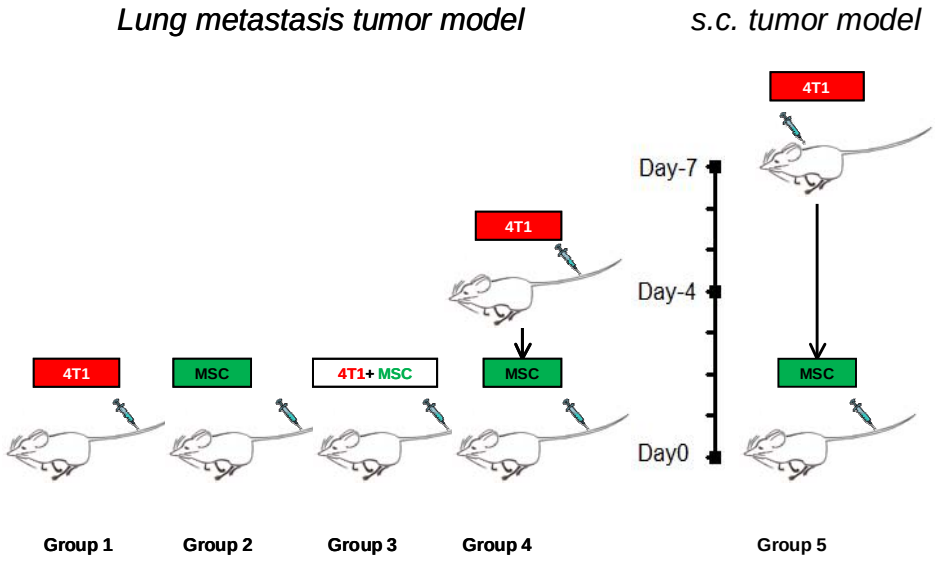


Fig. 3. Schematic illustration of the study design of MSC tumor tropism. Thirty Balb/C mice (age 6-8 weeks, weight 18-20g) were divided into 5 groups (n=6/group). group 1, tail vein injection of 2 x10^5 4T1 murine breast cancer cells to Balb/C mice and followed the tumor growth without administration of MSCs; group 2, Balb/C mice administered with MSCs only; group 3, 4T1 cells and MSCs were intravenously co-injected; group 4, MSCs were administered 4 days after tail vein injection of 4T1 cells; group 5, MSCs were administered 7 days after subcutaneous inoculation of 10^6 4T1 cells. Mice were imaged at multiple time points by BLI.

The fate of MSC-fLuc-GFP cells after intravenous injection to breast cancer lung metastasis (**Fig. 4**) and subcutaneous tumor models (**Fig. 5**) were monitored by bioluminescence imaging (BLI) using D-luciferin as the substrate. The 4T1-rLuc-mRFP tumor growth in the lung cavity was monitored by imaging rLuc expression using coelenterazine as the substrate. There was no cross-reactivity between D-luciferin and coelenterazine substrates (group 1). Administration of MSCs had little to no effect on the 4T1 tumor growth. In normal Balb/C mice, MSCs after intravenous injection were initially trapped within the pulmonary capillaries and subsequently died out (group 2). When MSCs were co-injected with 4T1 tumor cells, the MSC fLuc bioluminescence intensity was initially decreased and then gradually increased with time (group 3), suggesting that some of the MSCs survived and proliferated in the lung metastasis lesion sites. To further test the tumor homing properties, we injected MSCs at 4 days after 4T1 injection via the tail vein (group 4). The number of MSCs survived from group 4 is significantly lower than group 3. It is also of note that intravenous injection of MSCs to subcutaneous tumor model resulted in initial trapping of MSCs in the lung capillary followed by small number of MSCs homing to the subcutaneous tumor. MSCs homed and survived in the subcutaneous tumor also proliferated with time (group 5).

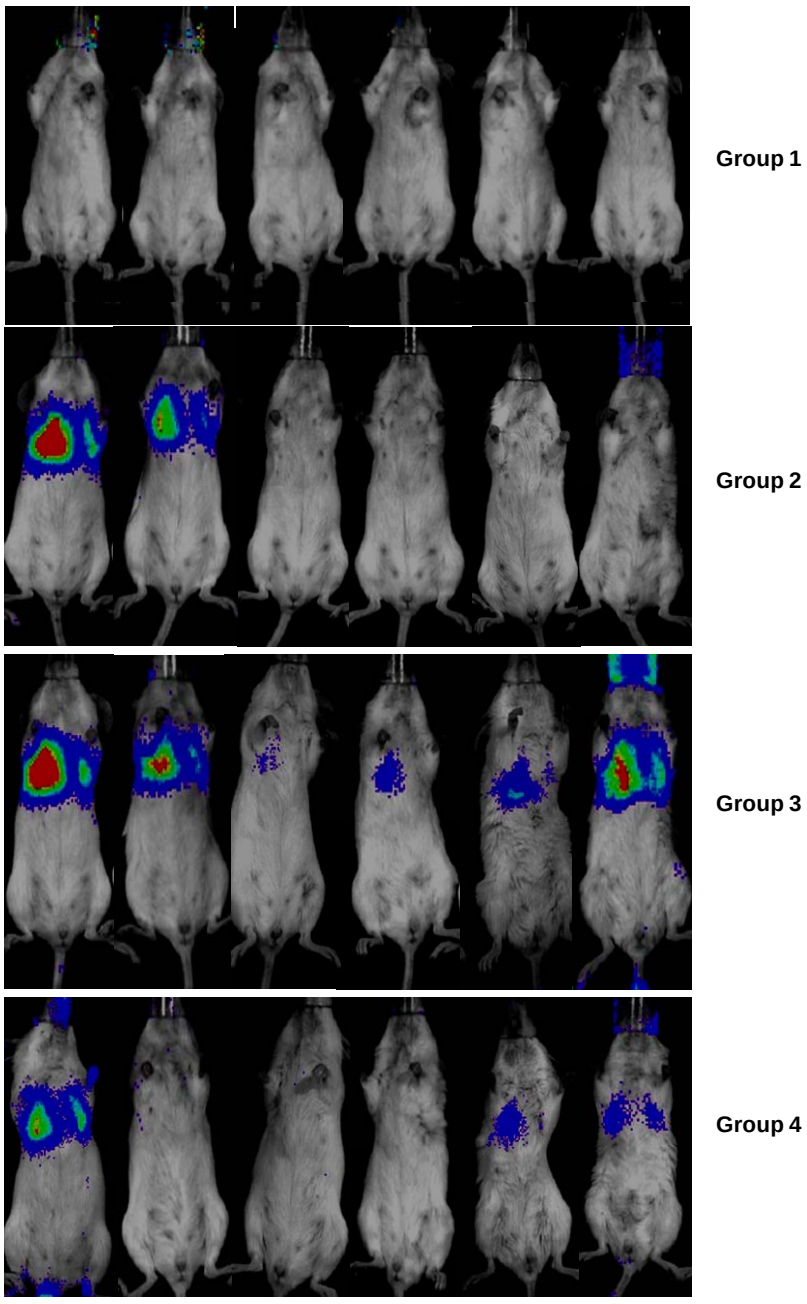


Fig. 4. Trafficking the fate of MSC-fLuc-GFP cells in 4T1-rLuc-mRFP tumor lung metastasis model. Four groups of mice (n = 6/group) were imaged. In group 1, tumor mice were injected with fLuc substrate D-luciferin and no BLI signal was detected, suggesting no cross-reactivity of D-luciferin with rLuc. In group 2, non-tumor bearing mice received i.v. injection of 5×10^5 MSCs. fLuc activity was only detected in lung area up to 1 day after MSC injection. In group 3, MSC and 4T1 cells were co-injected i.v. BLI imaging showed that fLuc activity dropped to minimum level at day 4, then increased gradually, peaked at day 11. In group 4, mice received i.v. injection of 4T1 followed by MSC cells in 4 days. BLI imaging showed that fLuc signal dropped to the lowest level at 1 day p.i., and did not increase until 9 day p.i.

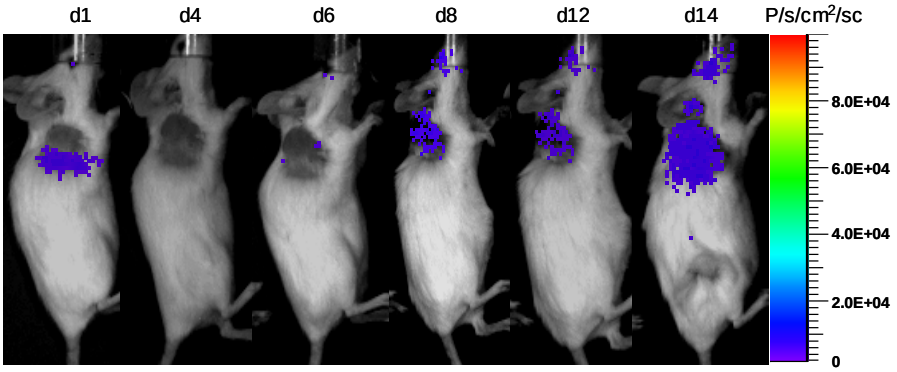
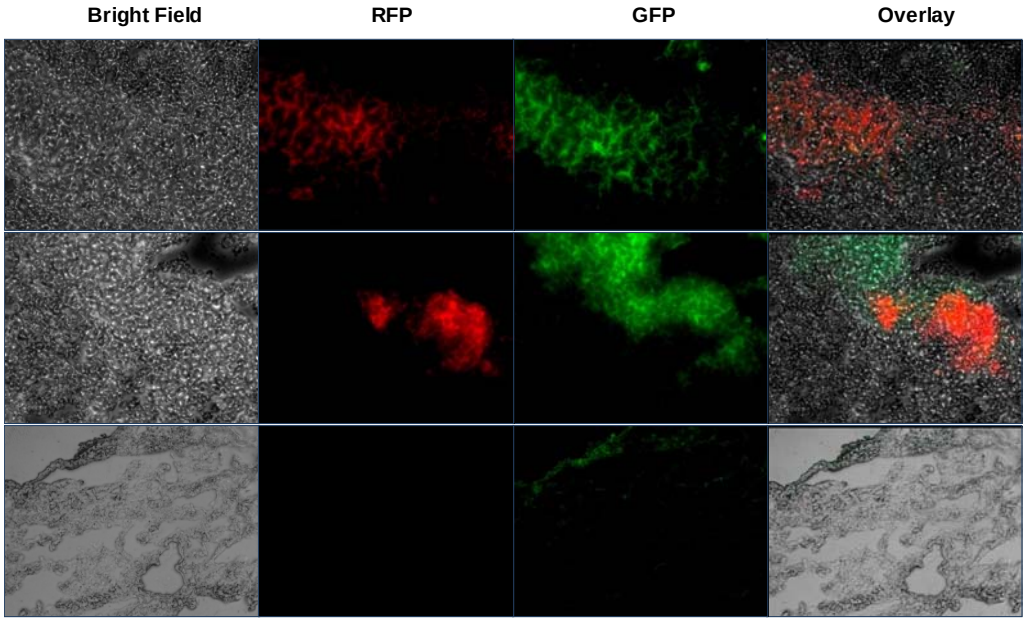


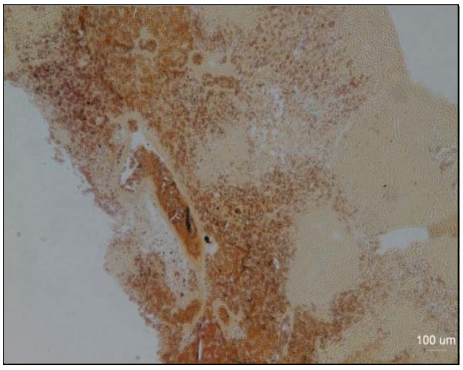
Fig. 5. Trafficking the fate of MSCs in subcutaneous 4T1-rLuc-mRFP tumor-bearing mice. MSC-L2G85 cells were intravenous injected at 1 week after 4T1 tumor inoculation and BLI signal was detected in the presence of fLuc substrate D-luciferin. The BLI signal in the tumor was detectable at 6 day p.i., which increased gradually afterwards, peaked at 14 day p.i. Note that the signal at day 1 is coming from the lung cavity.

MSCs in the lung tissue co-localize with the tumor cells (**Fig. 6**). MSCs in the lung metastasis niche were differentiated into osteoblasts instead of adipocytes (**Fig. 7**). MSCs in the subcutaneous tumor (**Fig. 8**) were differentiated into adipocytes rather than osteoblasts (**Fig. 9**). The gene expression profiles of MSCs influenced by tumor microenvironment was shown in **Fig. 10**. In s.c. 4T1 tumor, downregulated TGFβ1 activity may lead to a decrease of MSC-derived new osteoblasts and an increase in the formation of new adipocytes. In the lung metastasis model, we did not identify critical soluble growth factors responsible for enhanced MSC osteoblastogenic differentiation, however, we did find that the total proteins extracted from lung metastasis upregulated pro-osteogenic transcription factor RUNX2 expression. In the future, we will further investigate more molecules in the BMP/TGF pathway in the lung metastasis model, such as Smads, BMPRs and TGFβRs, etc. We will also investigate other signal pathways (e.g. Wnt) in this tumor model.

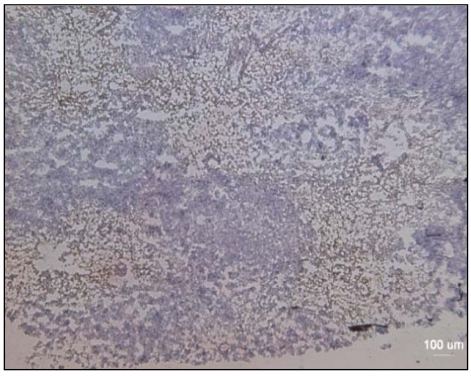


Upper: 4T1+MSC; Middle: 4T1 then MSC; bottom: normal lung + MSC

Fig. 6. Fluorescence microscope imaging of postmortem tissue sections confirmed the presence of MSCs in 4T1 tumor lung metastasis lesions in group 3 (upper) and group 4 (middle) mice. In non-tumor bearing mice received MSC injection (group 2, bottom), only very small number of GFP positive MSC cells were found in the lung tissue.



Alizarin Red S positive



Oil Red O staining negative

Fig. 7. Osteoblastogenic differentiation of MSCs in 4T1 lung metastasis model. Lung section prepared from the mice received i.v. injection of 4T1 cells followed by MSC injection were positive for Alizarin red S staining, and negative for Oil red O staining, which suggests calcium deposit (brown-red staining) between the tumor nodules, but no mature adipocytes. The lung tissue slices of non-tumor bearing mice after MSC injection were negative for both Alizarin Red S and Oil red O, owing to the inability of MSCs to survive in the normal lung area.

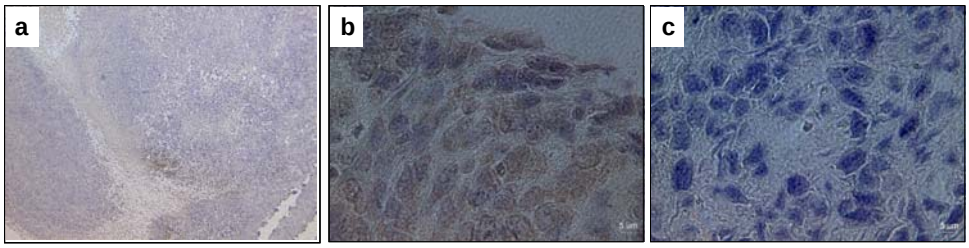


Fig. 8. Colocalization of MSCs and 4T1 cells in s.c. tumor model. To detect the residence of MSCs in s.c. tumor model, immunohistochemistry (IHC) was performed by using mouse anti-GFP antibody as the primary antibody and HRP-anti-mouse IgG as the secondary antibody. (**a**) GFP positive brown stained cells sporadically distributed throughout the tumor (100x). (**b**) High magnification field (400x) view of the MSC rich area. (**c**) No GFP+ cells were observed in the tumor slices without MSC injection.

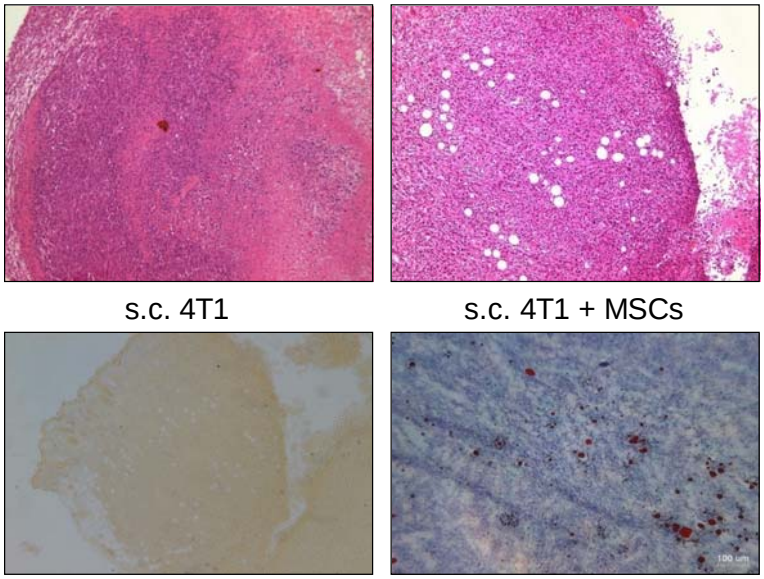


Fig. 9. Adipogenic differentiation of MSCs in s.c. 4T1 tumor model. H&E staining showed that there were multiple lipid vacuoles-like structures in tumor samples taken from the mice in group 5 (upper right) but not in those from without MSC injection (upper left). Tumor samples taken from group 5 were Oil Red O staining positive (red-stained lipid vacuoles, lower right) and Alizarin Red S staining negative (lower left), suggesting that MSCs localized to s.c. tumor differentiate into adipocytes but not osteoblasts.

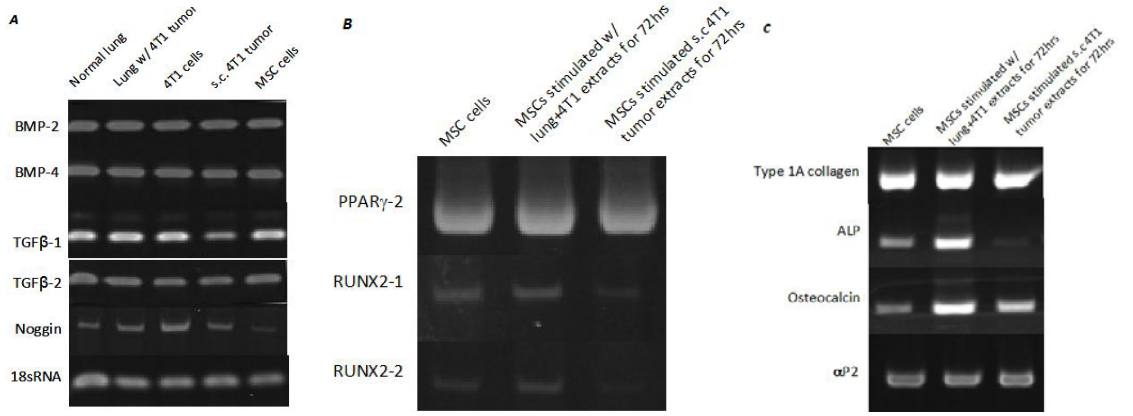


Fig. 10. Mechanism of dissimilar differentiation potential of MSCs in the lung metastasis and s.c. tumors. (***A***) RT-PCR analysis of gene expression profile. Decreased TGFβ1 mRNA level in s.c. 4T1 tumor sample might be responsible for a decrease of MSC-derived new osteoblasts and an increase in the formation of new adipocyte. (***B***) MSCs stimulated with protein extracts from lung tumor had increased mRNA level of pro-osteogenic transcription factors RUNX2-1. In contrast, MSCs stimulated with s.c. tumor extracts had decreased RUNX2-1 mRNA level. (***C***) MSCs stimulated with protein extracts from lung tumor for 72 h expressed high level of osteoblast characteristic gene ALP and osteocalcin, but not type IA collagen, as compared with untreated MSCs. In contrast, MSCs stimulated with protein extracts from s.c. tumor for 72 h had lower mRNA level of ALP. The mRNA level of osteocalcin also increased to some extent as compared with untreated MSCs.

KEY RESEARCH ACCOMPLISHMENTS

- We have successfully isolated murine mesenchymal stem cells (MSCs) from mice bone marrow;
  We have successfully transfected tumor cells and MSCs with different reporter genes and monitored the tumor growth and the fate of MSCs in vivo;
- We have successfully demonstrated that MSCs home to both subcutaneous breast cancer and its lung metastasis;
- We have shown that lung metastasis tropism is preferred over the subcutaneous tumor model and that MSC homing to premature lung metastasis is preferred over well-established lung metastasis;
- We have found that MSCs homing to lung metastasis differentiate into osteoblasts and those to subcutaneous tumor differentiate into adipocytes;
- We have unveiled the mechanism of MSC differentiation under different tumor environment.

REPORTABLE OUTCOMES

Abstracts:

Wang H, Cao F, De A, Gambhir S, Wu J, Chen X
Trafficking the fate of mesenchymal stem cells in vivo
55th SNM Annual meeting, New Orleans, LA June 2008

This abstract won the First Place Award in the Molecular Imaging Abstract track from the Society of Nucl Medicine (SNM) Molecular Imaging Center of Excellence. The same abstract was also chosen as an especially newsworthy abstract highlight at SNM news conference.

Wang H, Cao F, De A, Cao YA, Contag CH, Gambhir SS, Wu JC, Chen X
Trafficking the fate of mesenchymal stem cells in vivo
Manuscript submitted to the journal *Stem Cells*.

CONCLUSIONS

In conclusion, we have successfully demonstrated the ability of mesenchymal stem cells (MSCs) to home to both subcutaneous breast cancer and its lung metastasis. The microenvironment determines the tumor homing, differentiation and proliferation pattern of MSCs. We have thus accomplished aim 1 and are now ready to move to aim 2 to look at the capability of MSCs as delivery vehicles for breast cancer therapy.

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APPENDICES

None.